

Best Available Copy

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ : A61K 48/00, C12Q 1/68, C07H 21/02, 21/04</p>	<p>A1</p>	<p>(11) International Publication Number: WO 99/38537 (43) International Publication Date: 5 August 1999 (05.08.99)</p>
<p>(21) International Application Number: PCT/US99/02116 (22) International Filing Date: 29 January 1999 (29.01.99) (30) Priority Data: 60/073,065 30 January 1998 (30.01.98) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/073,065 (CON) Filed on 30 January 1998 (30.01.98) (71) Applicant (for all designated States except US): JOHN HOPKINS UNIVERSITY [US/US]; Suite 2-100, 2024 E. Monument Street, Baltimore, MD 21205 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): IMANI, Farhad [US/US]; 1118 Chatelaine Drive, Fallston, MD 21047-2346 (US). (74) Agent: BEATTIE, Ingrid, A.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published With international search report.</p>
<p>(54) Title: METHODS FOR TREATING Th1-ASSOCIATED IMMUNE DISORDERS (57) Abstract The invention encompasses methods of inhibiting an undesired Th1-mediated immune disorder, such as a Th1-mediated autoimmune disease, in a mammal suffering from or at risk of developing such a disorder by administering to the mammal a substantially pure dsRNA.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

METHODS FOR TREATING Th1-ASSOCIATED IMMUNE DISORDERSBackground of the Invention

The invention encompasses the field of IgE-mediated diseases and autoimmune diseases.

T helper cells which are CD4⁺ are subdivided into subsets based on profiles of cytokine production. T helper type 1 (Th1) cells produce interleukin-2 (IL-2), interferon- γ (IFN- γ), tumor necrosis factor- β (TNF- β), tumor necrosis factor- α (TNF- α), granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin-3 (IL-3), while T helper type 2 (Th2) cells produce interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-9 (IL-9), interleukin-10 (IL-10), TNF- α , GM-CSF, and IL-3. A number of diseases have been associated with an imbalance between these two T helper cell subsets.

Summary of the Invention

The invention provides a method of inhibiting an undesired Th1-associated immune response in a mammal, e.g., a Th-1 mediated autoimmune disease, by administering a substantially pure double-stranded ribonucleic acid (dsRNA) to the mammal. For example, the method involves identifying a mammal suffering from or at risk of developing an undesired Th1-mediated immune response and then administering a dsRNA composition to the mammal to prevent or treat a clinical disorder associated with the undesired immune response. The methods are also used to prevent onset of such a disease, and in the case of a recurring autoimmune disease, to prevent remissions. The autoimmune disease is a preferably characterized by chronic or recurring inflammation, e.g., an autoimmune disease which is Th1-mediated. Such diseases, e.g., Grave's disease, multiple sclerosis, Crohn's disease, rheumatoid arthritis, type 1 diabetes mellitus, and juvenile chronic arthritis, are characterized by an imbalance in Th1/Th2 cell responses

- 2 -

in the mammal in which Th1 response dominate. The methods are also used to inhibit transplant rejection, e.g., rejection of histoincompatible cells, tissue, or whole organs, which is mediated by an undesired Th1 immune responses. An undesired immune response is one that contributes to a clinically relevant disorder. For example, an undesired Th1-associated immune response is a pathological imbalance between the Th1 and Th2 helper cell subsets. Other undesired Th1-mediated immune responses include inflammatory reactions associated with Lyme Disease-associated arthritis, skin contact dermatitis, and Hashimoto's thyroiditis. Preferably, the mammal is a rat, mouse, guinea pig, hamster, dog, cat, pig, cow, goat, sheep, horse, monkey, or ape; more preferably, the mammal is a human.

dsRNA is administered to the mammal in a therapeutically-effective amount. A therapeutically-effective amount is one that induces a Th2 response in the mammal.

Induction of a Th2 response in the mammal antagonizes the undesired Th1 response which is involved in the pathogenic state, thereby preventing or reducing the severity of the pathogenic state. A Th1 associated immune response is inhibited by increasing a the level of a Th2 response, e.g, by increasing the level of Th2 cells in the mammal, by increasing the production of Th2-type cytokine production, by increasing the level of antibody production, or by inducing class switching of antibody isotype (e.g., switching from IgG or IgM production to IgE production). A Th1-associated immune response is also inhibited by decreasing the level of Th1 cells or decreasing the production of Th1-type cytokines.

By a "substantially pure dsRNA" is meant a dsRNA which is separated from those components (proteins and other naturally-occurring organic molecules) that naturally accompany it or a dsRNA which is chemically

- 3 -

synthesized. The dsRNA polymer is preferably at least 10 nucleotides, more preferably at least 30 nucleotides, more preferably at least 50 nucleotides, and most preferably at least 100 nucleotides in length. For example, the dsRNA to be administered is a 500 or 600-mer. At least 50%, more preferably at least 85%, more preferably at least 95%, more preferably at least 99%, and most preferably 100% of the RNA polymer composition to be administered is double-stranded. The therapeutic nucleic acid preparation may be delivered encapsulated in a cationic lipid preparation or unencapsulated.

The dsRNA is derived from a virus or virally-infected cell or is chemically synthesized. In the former case, the virus is preferably selected from the group consisting of a Respiratory Enteric Orphan Virus (reovirus), rhinovirus, vaccinia virus, adenovirus, influenza virus, Polio virus, Epstein-Barr virus, and bacteriophage ϕ . In the latter case, the dsRNA preferably contains a polyriboinosinic-polyribocytidilic acid (poly I:C), or a polyriboinosinic acid/polycytidilic acid/uridylic acid (poly(I):poly(C₁₂U)). Alternatively, the dsRNA is a synthetic viral dsRNA. A synthetic viral dsRNA is a chemically synthesized dsRNA which has the nucleotide sequence of a naturally-occurring dsRNA in a virion or a virally-infected cell.

The therapeutic composition is administered systemically or locally. For systemic administration, the preferred route is intravenous; however, in some cases oral, buccal, parenteral or rectal administration are used. For some autoimmune diseases such as arthritis, the dsRNA is administered locally to a site in the body of the mammal, e.g., an arthritic joint.

The invention also includes a method of determining a cellular response to a viral infection by measuring dsRNA-activated antiviral protein kinase (PKR) activation, e.g., by detecting measuring

- 4 -

autophosphorylation of PKR. An increase in autophosphorylation of PKR in a patient-derived sample of cells compared to the amount of autophosphorylation in a sample of cells known to be uninfected (or a standard control value) indicates an allergic or asthmatic response to the viral infection. Rather than measuring autophosphorylation of PKR in the patient-derived sample of cells, expression of germline ϵ is measured. An increase in the level of expression of germline ϵ in a patient-derived sample of cells compared to the amount of expression in a sample of cells known to be uninfected (or a standard control value) indicates an allergic or asthmatic response to the viral infection.

A method of predicting an asthmatic attack in a mammal is also within the invention. PKR activation is measured by detecting autophosphorylation of PKR. An increase in autophosphorylation of PKR in a sample of patient-derived cells (compared to a normal control sample of cells or a standard control value) indicates increased risk of the onset of an asthma attack in the patient.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figs. 1A-B are autoradiographs of the products of a polymerase chain reaction (PCR) on an electrophoretic gel showing that rhinovirus infection of Ramos B cells leads to the expression of germline ϵ transcript. Fig. 1A shows germline ϵ expression, and Fig. 1B shows glyceraldehyde phosphate dehydrogenase (GAPDH) expression as a control for equal loading of RNA in each lane.

Fig. 2 is a photograph of PCR products on an electrophoretic gel showing that rhinovirus mRNA was detected in rhinovirus-infected B cells.

- 5 -

Figs 3A-B are photographs of PCR products on an electrophoretic gel showing that infection of Ramos B cells with respiratory syncytial virus (RSV) results in expression of germline ϵ . Fig. 3A shows expression of germline ϵ , and Fig. 3B shows expression of the housekeeping gene, GADPH, as a control.

Fig. 4 is a photograph of a BglI digested germline ϵ PCR fragment.

Figs. 5A-B are autoradiographs showing that vaccinia virus-encoded E3L polypeptide modulates germline ϵ expression. Fig. 5A shows expression of germline ϵ , and Fig. 5B shows expression of GADPH as a control.

Fig. 6 is an autoradiograph showing the results of a Western blot assay in which vaccinia virus proteins were detected in infected B cells.

Fig. 7 is an autoradiograph showing that E3L-deleted vaccinia virus activates PKR protein expression in Ramos cells.

Figs. 8A-B are autoradiographs showing that dsRNA treatment of B cells induced the expression of germline ϵ . Fig. 8A shows expression of germline ϵ , and Fig. 8B shows expression of GADPH as a control.

Figs. 9A-B are photographs of PCR products on an electrophoretic gel showing that germline ϵ expression induced by dsRNA is not mediated by interferon. Fig. 9A shows expression of germline ϵ , and Fig. 9B shows expression of GADPH as a control.

Fig. 10 is an autoradiograph of the results of an in vitro kinase reaction showing that PKR was induced in an inactive form by IFN- α and IFN- β , but not by IFN- γ .

Fig. 11 is an autoradiograph of an electrophoretic mobility shift assay (EMSA) showing the kinetics of dsRNA-induced NF κ B activation.

Fig. 12 is an autoradiograph of an EMSA showing that p50 and p65-containing NF κ B complexes were induced by dsRNA.

- 6 -

Fig. 13 is a photograph of PCR products on an electrophoretic gel showing that dsRNA treatment induces cytokine mRNA in human peripheral blood lymphocytes.

Fig. 14 is a bar graph showing that IL-4 is
5 induced by dsRNA treatment.

Detailed Description of the Invention

Th1 cells and Th2 cells are mutually antagonistic. The invention provides methods of treating or preventing Th1-mediated autoimmune diseases by administering dsRNA.
10 Many of the Th1-mediated autoimmune disease are characterized by chronic inflammation with strong cell-mediated immunity and a low antibody response. Modulation of the Th1 bias in chronic inflammatory autoimmune diseases such as multiple sclerosis,
15 rheumatoid arthritis, and diabetes, to increase Th2 responses is clinically beneficial.

Effective treatments for autoimmune diseases have been elusive. Often, therapeutic approaches are limited to treating symptoms rather than the cause of the
20 disease. The elucidation of the pathway for IgE induction, and the and the exploitation of this pathway to induce a Th2 response provides a solution to a longfelt problem in the treatment of Th1-mediated autoimmune diseases. By restoring the balance between
25 the Th-1 and Th-2 cytokine response with administration of dsRNA, proper immune function is restored.

Viral infections induce IgE class switching. The mechanism by which this occurs involves accessibility of viral dsRNA, leading to autophosphorylation of PKR, which
30 in turn induces both p50 homodimerization and p50/p65 heterodimerization of NKκB. This favors a Th2 cytokine profile (thereby antagonizing the Th1 response) and IgE production.

The dsRNA to be administered includes, but is not
35 limited to dsRNA purified from a virus, e.g., Respiratory Enteric Orphan Virus (REO). Viral dsRNA is isolated

- 7 -

using well known methods, e.g., from virions or from virus-infected cells. Eukaryotic viruses such as Reovirus or Rotavirus or prokaryotic viruses such as bacteriophages (e.g., bacteriophage ϕ such as bacteriophage ϕ 6) are used as sources of dsRNA. Virions are isolated using known methods, and dsRNA is purified from those components (proteins and other naturally-occurring organic molecules) which naturally accompany it in the virion or in an infected cell by standard phenol/chloroform extraction and ethanol precipitation (e.g., using methods described in Drastini et al., 1992, J. Virological Methods 39:269-278; Onodera et al., 1995, Virology 212:204-212; and Mindich L., 1988, Adv. in Virus Research 35:137-176). Purity of the dsRNA preparation is determined spectrophotometrically, e.g., at 260/280 nm. Alternatively, purity is assessed by resolving the components of the preparation using SDS-PAGE followed by silver staining, a procedure that allows visualization of dsRNA and contaminating proteins and DNA. A preparation of virally-derived RNA is substantially pure when it is at least 50%, preferably at least 75%, more preferably at least 90%, more preferably at least 95%, and most preferably 100% RNA.

dsRNA is also generated synthetically using methods well known in the art. Poly I:C is chemically synthesized or it can be purchased. Other ribonucleotide polymers are chemically synthesized using known methods. For example, a synthetic ribonucleotide polymer is synthesized to mimic the nucleotide sequence of a naturally-occurring viral dsRNA or it may contain non-naturally occurring nucleotides. In addition to adenylic acid, guanylic acid, cytidylic acid, and uridylic acid, artificial or non-naturally occurring nucleotides are incorporated into an RNA polymer if desired. The dsRNA may be matched, e.g., poly (I:C), or mismatched, e.g., poly (I):poly (C₁₂U).

- 8 -

Th1-mediated autoimmune diseases to be treated include Grave's disease, multiple sclerosis, Crohn's disease, rheumatoid arthritis, type 1 diabetes mellitus, rheumatoid arthritis, and juvenile chronic arthritis.

5 Identifying patients with these autoimmune diseases is well known in the art.

Other Th1-associated immune disorders include transplant rejection. dsRNA is administered before and/or after transplantation to prevent or reduce the
10 severity of tissue rejection. The therapeutic method is also to prevent or reduce the severity of other chronic or acute inflammatory disorders in which Th1 immune responses are involved, e.g., Hashimoto's thyroiditis, Lyme Disease-associated arthritis, and contact
15 dermatitis. Methods of diagnosing these disorders are also well known in the art.

The invention also encompasses methods for exploiting the mechanism by which dsRNA leads to autophosphorylation of PKR, which in turn induces both
20 p50 homodimerization and p50/p65 heterodimerization of NK κ B. Such mechanisms favor a Th2 cytokine profile and IgE production. As a result, IgE-mediated human asthma and allergy are exacerbated. Inhibitors of dsRNA, e.g., reovirus sigma 3 protein and vaccinia E3L protein, are
25 used to treat patients suffering from asthma by blocking IgE production.

Therapeutic approaches to Th1-mediated autoimmune

dsRNA (synthetic or derived from virally-infected cells) is introduced into a patient suffering from a
30 Th1-associated immune disorder (or at risk of developing such a disorder) by standard nucleic acid delivery systems. Suitable nucleic acid delivery systems include microencapsulation (e.g., described in Tice et al. in European Patent Application EP 0 248 531 A2), liposomes,
35 receptor-mediated delivery systems, naked nucleic acid delivery, and vector-mediated delivery. For example, for

administration to human patients, dsRNA is stabilized with poly-L-lysine and carboxymethylcellulose.

The double-stranded portion of a dsRNA RNA polymer is preferably over the length of at least a 10-mer.

- 5 Preferably the length of the double-stranded portion is over at least 30 nucleotides, more preferably at least 100 nucleotides, and most preferably at least 500 nucleotides. Regardless of the length of the polymer, in preferred embodiments, the entire length of a dsRNA
- 10 polymer is double-stranded. The maximal length of is limited by the toxicity of the dsRNA polymer; toxicity is tested using methods well known in the art, e.g., those described by Cornell et al., 1976, J. Natl. Cancer Instit. 57:1211-1216 or Freeman et al., 1977, J. Med.
- 15 Virol. 1:79-93. Toxicity of parenterally-administered dsRNA is also related to the average molecular weight of the therapeutic composition; preferably, the average molecular weight of the dsRNA is less than 50,000 Da.

- dsRNA is administered in a pharmaceutically
- 20 acceptable carrier. Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to an animal, e.g., physiological saline. A therapeutically effective amount is an amount of the dsRNA of the invention which is capable of
- 25 producing a medically desirable result in a treated animal, i.e., induction of a Th2-type immune response. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the
- patient's size, body surface area, age, the particular
- 30 compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages will vary, but a preferred dosage for intravenous administration of dsRNA is from approximately 0.1 to 100 mg/kg of body weight.
- 35 As is described below (Example 14 and Fig. 14), dsRNA induces production of a Th2-type profile of cytokines

- 10 -

(and therefore a Th2 immune response) in the range of about 0.01 $\mu\text{g/ml}$ to 5 $\mu\text{g/ml}$; this effective concentration is equivalent to mg/kg doses. Doses in the range of 1 to 10 mg/kg of body weight (e.g., 3-12 mg/kg, e.g., 6 mg/kg) are safely administered to human patients with minimal toxicity. Alternatively, dsRNA is administered in a single dose (e.g., in the range of 100-1000 mg, e.g., 200-600 mg). The dsRNA may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously. However, in some situations, dsRNA may be administered directly to a target site, e.g., to an arthritic joint. The preferred form of the composition and route for delivery for administration depends on the intended mode of administration and therapeutic application.

Viral Modulation of Immune Cell Function

Viruses modulate cellular functions by two different enzymatic pathways. The first pathway is the 2'-5' oligoadenylate synthetase/RNase L pathway, and the second pathway is the PKR pathway. Both pathways are induced upon viral infection but remain inactive. Double-stranded RNA, viral or synthetic, can activate both IFN-induced pathways as long as it contains an uninterrupted minimum length of 30-100 base-paired double-stranded region. Since dsRNA is present at some stage during replication of most viral strains (RNA and DNA viruses), dsRNA is the signal by which cells recognize viral infection.

A cellular response to viral infection is measured by detecting PKR activation. Detection of PKR autophosphorylation is useful in predicting an allergic or asthmatic response in a patient. PKR activation is preferably measured by detecting PKR autophosphorylation as described in the examples which follow, but other methods known to those of skill in the art can also be used.

Oligo adenylate pathway

Although the 2'-5' oligoadenylate synthetase is induced 50-100 fold upon interferon treatment, it remains in an inactive form until it interacts with dsRNA. The enzyme 2'-5'adenylate synthetase catalyzes the polymerization of ATP into a series of heat-stable oligomers consisting of 2-15 molecules of adenylic acid that are linked in an unusual 2'-5' fashion. These series of 2'-5' oligo-adenylates are not directly involved in the inhibition of translation; rather they activate a latent endoribonuclease called RNase L (Latent). Activated RNase L has the ability to degrade single stranded RNA. Viral mRNA is degraded preferentially over cellular mRNA. This selective in vivo degradation of viral mRNA is due to localized activation of the synthetase only in compartments where viral replication is taking place.

Protein kinase pathway.

Anti viral protein kinase PKR (previously known as p1, p68, DAI and dsRNA-activated kinase) was originally described by Lebleu et al. in 1976 (Lebleu et al., 1976, Proc. Natl. Acad. Sci. USA, 73:3107-3111). A unique feature of this enzyme is that it is activated by dsRNA. One of the key mechanisms by which eukaryotic cells regulate cellular activity is by protein phosphorylation-dephosphorylation. PKR is a serine/threonine protein kinase with a molecular weight 67 kDa (mouse) or 72 kDa (human). It is present in untreated cells, but its level is increased five to ten fold after viral infection and exposure to IFN (predominantly IFN- α and IFN- β).

Results from in vitro assays suggest that PKR can be activated by low concentrations of dsRNA (0.001- 10 μ g/ml), but high concentrations (>10 μ g/ml) are inhibitory. Activated PKR, evidenced by autophosphorylation, subsequently phosphorylates the small (a) subunit of the eukaryotic protein synthesis

- 12 -

initiation factor 2 (eIF-2 a). This leads to inactivation of eIF-2 a and a block in the initiation of translation in the cellular compartment in which the viral infection takes place. The data described herein shows a direct
5 action of virus on the production of germline c transcripts.

NFκB activation

NFκB is a multisubunit transcription factor present in eukaryotic cells. The canonical complex is
10 comprised of a 50 kDa (p50, RB-1) and a 65 kDa (p65, Rel A) subunit. Other related subunits of the NFκB complex (p105, Rel B, Rel C) have also been described. Due to the association of IRB (the inhibitor of NFκB), this transcription factor is present intracytoplasmically in
15 an inactive form. Many different stimuli such as LPS, anti-CD40, various cytokines and dsRNA have been shown to activate NFκB. The activation of NFκB is mediated by phosphorylation and thereby inactivation and degradation of IRB. The activated NFκB complex, which contains
20 hetero- and homodimers of different subunits, migrates to the cell nucleus where it can bind to a decameric motif. This motif is associated with the enhancer region of many genes. The composition of different hetero- and homodimers is thought to differentially regulate
25 transcriptional activation of various genes. For example, a 40-fold decrease was detected in the production of antigen-specific IgE in p50 knockout mice.

In addition, NFκB may be important for IL-4 expression. Since IL-4 is a potent Th2 cytokine,
30 activation of PKR and subsequently NFκB by dsRNA could lead to Th2 responses.

Virally-encoded inhibitors of PKR

Activation of PKR is thought to be an almost universal signal for viral infections. The presence of
35 dsRNA as a genomic fragment, replicative intermediate, or dsRNA stem and loop structures can activate this

- 13 -

enzymatic pathway. However, to successfully replicate, many viral strains have evolved to encode inhibitors of this host defense mechanism. For example, cells infected with adenovirus contain a virally-encoded small molecule
5 of RNA called VA1. VA1 RNA is composed of 160 nucleotides with short double-stranded regions. VA1 binds to the protein kinase but the lengths of the double-stranded regions are not sufficient to activate this enzyme. Thus, binding of VA1 RNA to PKR blocks the
10 interaction of PKR with longer dsRNA.

Reovirus-encoded s3 protein also inhibits PKR. This inhibition is due to the interaction of s3 protein, in a stoichiometric manner, with dsRNA. In effect, s3 protein is capable of masking the dsRNA from PKR.
15 Vaccinia virus also contains an inhibitor of PKR. Vaccinia virus-encoded protein kinase inhibitory activity is due to the presence of the E3L polypeptide that also interacts in a stoichiometric manner with dsRNA. Several other viral strains such as HIV, Polio, Influenza, and
20 Epstein-barr virus have also been reported to contain PKR inhibitory factors. To date, no such inhibitors have been found for RSV or rhinovirus. Therefore, in infections with rhinovirus and RSV, PKR can be activated, and in turn, activate NK κ B. Although most viral strains
25 examined to date possess PKR inhibitory factors, PKR is still activated by infections with several viral strains. This apparent discrepancy may be due to the ratio between the level of the PKR inhibitory factor and dsRNA present in the infected cells. Alternatively, it is known that
30 subtype variability among viral strains may affect activation. For example, reovirus type 1 which produces high levels of s3 protein, can replicate much more efficiently than the reovirus type 3 which produces low levels of s3 protein. This difference in viral
35 replication is attributed to the ability of s3 protein to inhibit PKR activation.

- 14 -

As described below, infection of Ramos B cells with vaccinia virus lacking the E3L polypeptide (an inhibitor of PKR activation) resulted in induction of germline ϵ expression, while, infection with wild type vaccinia virus expressing E3L did not result in an increase in the expression of germline ϵ . These data indicate that induction of IgE class switching as a mechanism by which viral infection augments allergic asthma.

10 The examples described below are provided for illustrative purposes only and are in way intended to limit the scope of the present invention.

Cell lines, culture conditions and reagents

Human Burkitt's lymphoma B cell line (American Type Culture Collection (ATCC) designation number 15 2G6.4C10; "Ramos") was purchased from ATCC (Rockville, MD). Cells (1×10^5 to 1×10^6 /ml) were grown in RPMI-1640 supplemented with 10% fetal calf serum, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 20 gentamicin sulfate at 5 μ g/ml. Cells were cultured at 37°C in a 5% CO₂ humidified chamber. Synthetic dsRNA, e.g., polyriboinosinic-polyribocytidilic acid (poly I:C) was purchased from SIGMA, St. Louis, MO

25 Example 1: Rhinovirus infection of Ramos cells leads to the expression of germline ϵ transcript

Ramos cells (surface IgM⁺ uncommitted B cells) were infected with equal units (1 tissue culture infective dose/cell) of rhinovirus 14 or 16. After 48 hours, total cellular RNA was extracted and 1 μ g of RNA 30 was subjected to reverse transcription and 25 cycles of PCR using primers specific for germline ϵ . The PCR products were resolved on a 2% agarose gel. To determine the identity of a unique 210 bp band, southern blot hybridization was carried out using standard methods. 35 After gel electrophoresis, the PCR amplified fragments were transferred to nylon membrane (Immobilon-N,

- 15 -

Millipore). The membrane was probed with a ^{32}P -labeled oligonucleotide specific for the internal sequence of germline ϵ . After hybridization and washing, the products were visualized by autoradiography.

5 Both rhinovirus strains (14 and 16) induced germline ϵ expression. However, infection with rhinovirus 16 led to a lower level of germline ϵ expression than rhinovirus 14. It is conceivable that these two viral strains differ in their capability to
10 activate PKR. Alternatively, the difference may reflect titration discrepancies. Neither untreated or mock infected cells showed germline ϵ expression. A probe for IL-4, a potent stimulator of IgE class switching, was used as positive control, and a probe for GAPDH, a
15 housekeeping gene, was used to show equal loading of RNA in each lane. PCR fragments were visualized by ethidium bromide staining (Figs. 1A-B).

Example 2: Detection of rhinovirus mRNA in infected B cells

20 Since infection of human B cells with rhinovirus has not been previously demonstrated, experiments were undertaken to demonstrate the presence of rhinovirus RNA (14 and 16) in infected Ramos cells. RT-PCR was performed on 1 μg of total RNA using primers specific for
25 both rhinovirus strains. The PCR products were resolved on an electrophoretic gel, and were visualized by ethidium bromide staining. As shown in Fig. 2, the data indicate that rhinovirus RNA, both type 14 and 16, were detectable in the infected cells.

30 The presence of viral RNA in the cells does not necessarily imply replication. However, the activation of PKR does not require viral replication. To activate PKR, the positive stranded viral genome must be transcribed into a negative strand RNA. The association
35 of the negative strand with the genomic RNA leads to formation of dsRNA. The polymerization of the genomic

- 16 -

RNA into negative strand RNA is mediated by a viral particle-associated polymerase. Rhinovirus dsRNA is also present in rhinovirus infected human embryo lung cells. Members of the picornaviridae family (rhinovirus, poliovirus and mengovirus) can also activate PKR.

Example 3: Infection of Ramos B cells with RSV results in expression of germline ϵ

Ramos cells were infected with human RSV at a multiplicity of infection (M.O.I.) of 5 plaque forming units (pfu)/cell, and after 48 hours, total cellular RNA was extracted (n=2). To increase the amount of PCR products to a level detectable by ethidium bromide staining, the PCR cycle number was increased to 42. The PCR products were resolved by electrophoresis, and then were visualized by ethidium bromide staining (Figs. 3A-B).

Example 4: Restriction mapping of germline ϵ PCR fragment

The identity of a 210 PCR product as germline ϵ was confirmed by BglI digestion. The resulting bands were 95 and 115 bp as expected and are shown in Fig. 4. Not only does the BglI digestion confirm the identity of this PCR product as germline ϵ , it can also be used as an alternative to southern blot analysis to identify the band.

Example 5: Vaccinia virus-encoded E3L polypeptide modulates germline ϵ -expression

Two strains of vaccinia virus were used to study germline ϵ expression. Wild type Vaccinia virus (Copenhagen strain) inhibits the activation of PKR due to virally encoded polypeptide E3L. The E3L polypeptide inhibits PKR by interacting with dsRNA and blocking its interaction with PKR. The second strain, a mutant of vaccinia virus is identical to the wild type Copenhagen strain except that the sequences encoding the E3L polypeptide were deleted. Ramos cells were infected

with wild type vaccinia virus and the E3L-deleted mutant at a M.O.I. of 5 pfu/cell (n=3). RNA extraction, RT-PCR and gel electrophoresis of the PCR products were performed. As shown in Figs. 5A-B, the E3L-deleted mutant, known to activate PKR, also induced germline ϵ . In contrast, wild type vaccinia virus, which does not activate PKR, did not induce germline ϵ , suggesting that E3L regulation of PKR activity modulates IgE class switching.

10 Example 6: Detection of vaccinia virus proteins in infected B cells

To determine whether vaccinia virus could infect Ramos B cells, a Western blot analysis was carried out using rabbit polyclonal anti-vaccinia virus p25 protein. 15 Equal amounts (as determined by bicinchoninic acid protein determination assays, Pierce, Rockford, IL) of detergent extracts prepared from vaccinia virus infected cells (1×10^6) were subjected to SDS-PAGE and Western blot analysis. The immunoblotted proteins were 20 visualized using enhanced chemiluminescence assay (ECL; Amersham Corp. Arlington Heights, IL). As shown in Fig. 6, vaccinia virus-encoded p25 is expressed in human B cells infected with the wild type and the E3L-deleted mutant virus.

25 Example 7: E3L-deleted vaccinia virus activates PKR in Ramos cells

To determine whether vaccinia infection of B cells leads to activation of PKR, Ramos cells were infected with the virus (previous studies were limited to HeLa 30 cells). After 24 hours of infection, detergent lysates were prepared and equal amounts (from 5×10^5 cells) were subjected to *in vitro* kinase reactions using [γ - 32 P]-ATP in the absence or presence of 1 μ g/ml of dsRNA (n=2). The proteins were resolved by 10% SDS-PAGE and were 35 visualized by autoradiography of the dried gel. The band at approximately 72 kDa is PKR. These results

- 18 -

demonstrate that, as with HeLa cells, infection of Ramos cells with the E3L-deleted vaccinia virus results in activation of PKR. As indicated in Fig. 7, addition of exogenous dsRNA to cell extracts prepared from E3L-deleted infected cells did not result in any increase in PKR activation. This is likely due to maximal activation of PKR by E3L-deleted vaccinia virus infection.

The common molecular mechanisms by which viral infections induce and exacerbate asthma are not yet clear. Additionally, animal studies examining viral induction of IgE production do not provide data indicating which cells mediate the virally induced effects. Finally, it is not clear from data collected in animal studies, whether the virally-induced increase in IgE levels is due to IgE class switching or to activation of IgE-committed cells. In contrast to previous studies, the data described herein was conducted in a human system and demonstrates a direct viral modulation (e.g., by dsRNA) of surface IgM⁺ B cells results in IgE class switching.

Example 8: dsRNA treatment induces expression of germline ϵ

Contacting cells with dsRNA mimics the effects of viral infection with respect to the activation of PKR. Therefore, the effects of dsRNA on IgE class switching was examined. Since the first step in IgE class switching is the expression of an immature IgE transcript (germline ϵ), RT-PCR was carried out to detect a germline ϵ transcript as an indication of IgE class switching.

Ramos cells were either left untreated or were treated with various concentrations of dsRNA. After 72 hrs, total cellular RNA was extracted and equal amounts were subjected to RT-PCR using primers specific to germline ϵ . RNA was isolated by TRIzol total RNA isolation system (Bethesda Research Laboratories (BRL), Gaithersburg, MD). After reverse transcription, the cDNA

- 19 -

was amplified in the presence of 2 µg/ml of primers, 100 µM dNTPs, 0.25 U of Taq polymerase (Perkin Elmer), 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, and 0.001% gelatin in a final volume of 25 µl. Primers for constant
5 ε exon-derived sequence (5' AGAGGTCGGGCATTGGAGGGAATGT 3'; SEQ ID NO:1) and germline ε exon-derived sequence (5' AGGCTCCACTGCCCCGGCACAGAAAT 3'; SEQ ID NO:2), and GAPDH forward primer (5' CACAGTCCATGCCATCACTG 3'; SEQ ID NO:3) and reverse primer (5' TACTCCTTGGAGGCCATGTG 3'; SEQ ID
10 NO:4) were used in the PCR reactions. PCR was performed in a DNA thermal cycler (Perkin-Elmer) for 42 cycles for vaccinia infections, 25 cycles for rhinovirus infection or for germline ε, and 25 cycles for GAPDH. For restriction endonuclease mapping, the 210 bp PCR product
15 corresponding to germline ε cDNA was purified using the QIAquick gel extraction kit (Qiagen, Chatsworth, CA). The purified fragment was digested with BglI enzyme (BRL) for 2 hrs at 37°C, and the products were resolved on a 2% agarose gel. A 100 bp ladder (BRL) was used to provide
20 molecular weight markers. To detect any differences in the induction of germline ε transcript by the two viral strains of rhinovirus used, the amplified products after 25 cycles were visualized by southern blot hybridization using a [γ -³²P]-end labelled internal primer specific to
25 germline ε (5' AGCTGTCCAGGAACCCGACAGGGAG 3'; SEQ ID NO:5).

Data revealed that treatment of Ramos cells with dsRNA resulted in a concentration dependent expression of germline ε transcript (Figs. 8A-B). A unique 210 bp PCR
30 product was purified and subjected to restriction enzyme mapping using BglI. The resulting fragments were 95 and 115 bp in length corresponding to the appropriate expected sizes for BglI digest of germline ε cDNA sequence.

35 Example 9: Germline ε expression induced by dsRNA is not mediated by interferon

- 20 -

dsRNA treatment of eukaryotic cells results in the elaboration of IFNs. Experiments were therefore undertaken to determine whether the dsRNA-induced IgE class switching is due to the autocrine effects of IFNs on Ramos cells. Ramos cells were treated with 100 u/ml of human IFN- α or IFN- β (Lee Biomolecular, San Diego, CA), IFN- γ and human IL-4 (Sigma, St. Louis, MO). After 24 hrs, cells were washed twice with isotonic buffer containing, 20 mM Hepes, pH 7.5, 120 mM KCl, 5 mM MgOAc and 1 mM DTT. Cells were then lysed in buffer containing 20 mM Hepes, 120 mM KCl, 5 mM MgOAc, 1 mM Benzamidine, 1mM DTT and 1% Nonidet P-40. After RNA extraction and RT-PCR using germline ϵ -specific primers (described above), the data revealed that IFN treatment alone did not induce class switching in Ramos cells (Figs. 9A-B). However, RT-PCR on the RNA extracted from cells treated with IL-4, a cytokine known to be a potent inducer of IgE class switching, amplified the 210 bp product corresponding to germline ϵ .

20 Example 10: PKR is induced in an inactive form by IFN- α and IFN- β but not IFN- γ

To determine whether IFN treatment was effective in the induction of PKR, detergent cell extracts from the mock-treated or IFN-treated cells were prepared.

25 Mixtures for in vitro phosphorylation of cellular extracts contained 20 mM Hepes, pH 7.5, 90 mM KCl, 5 mM MgOAc, 1 mM DTT, 100 μ M [γ - 32 P]ATP (Amersham, specific activity 1 Ci/mM), 100 μ M ATP (Sigma, St. Louis, MO), and equal amounts of detergent extract prepared from 1×10^6 cells, in a final volume of 25 μ l. dsRNA was added to the reaction mixtures, and the mixtures were incubated at 30°C. After 10 min, the reactions were quenched by adding SDS-sample buffer containing a final concentration of 2.5% β -mercaptoethanol and boiling for 2 min. The

35 reduced, denatured proteins were then resolved using 10% SDS-PAGE and visualized by autoradiography.

The results of *in vitro* kinase reactions, performed in the presence or absence of dsRNA, showed that both IFN- α and IFN- β could increase the expression of PKR in an inactive state, but PKR was only activated in the presence of dsRNA. These data indicate that the induction of PKR without activation by dsRNA is not sufficient for induction of a Th2 type response such as class switching (Fig. 10). Treatment of Ramos cells with IFN- γ , however, did not result in such an increase (Fig. 10).

Example 11: Activation of NF κ B in Ramos cells by dsRNA treatment

dsRNA as well as viral infections activate NF κ B through the activation of PKR and subsequent phosphorylation and inactivation of IRB. To examine the effects of dsRNA treatment on NF κ B activation in Ramos cells, EMSAs were carried out.

Ramos cells were treated with 10 μ g/ml of dsRNA and whole cell extracts were prepared after various times post treatment. Cell extracts for EMSA were prepared using standard methods. EMSAs were performed using [γ - 32 P]-end labelled NK κ B (from kappa light chain) consensus oligonucleotide (Promega, Madison, WI). The reactions (20 μ l) contained of 2 μ L of nuclear extract in buffer containing 20 mM Hepes (pH 7.5), 50 mM KCl, 0.2 mM EDTA, 10% glycerol, 40 μ g/ml Poly dI.C: dI.C, 0.05% NP-40 and 0.5 μ l of labeled probe. After 15 min at 37°C, the protein/DNA complexes were resolved on 4.5% non-denaturing polyacrylamide gel and were visualized by autoradiography of the dried gels.

Data from EMSA showed that NF κ B complex was induced upon dsRNA treatment (Fig. 11). The maximal level of NF κ B activation was observed at 4 hrs post treatment.

Example 12: p50 and p65 containing NK κ B complexes were induced by dsRNA

- 22 -

To determine which of the NF κ B subunits were involved in the dsRNA-induced complex, supershift assays were performed using mAbs to several known subunits of NF κ B complex. Ab-mediated supershifts revealed that both p50 (RB-1) and p65 (Rel-A) subunits were induced upon dsRNA treatment (Fig. 12), however there was no supershift with Ab to c-Rel. Also, the combined addition of anti p50 and anti p65 induced the retardation of p65-containing complex without any further change in the retardation of p50-containing complex, suggesting the presence of homodimer of p50 as well as heterodimer of p50 and p65 in the dsRNA treated Ramos cells.

Example 13: IL-4 mRNA is induced by dsRNA treatment of human cells

To determine whether dsRNA treatment could induce cytokine expression, Ramos B cells and peripheral human blood lymphocytes (PBL) were treated with dsRNA. Semi-quantitative RT-PCRs were performed for IL-4, IFN- γ , IL-12 (p35 subunit), IL-12 (p40), IL-13, and the housekeeping gene GAPDH. The cycle numbers were optimized in order to harvest the products within the linear range of amplification.

At high concentrations of dsRNA (30 μ g/ml), IL-4 mRNA expression was inhibited. The data also shows that the activation of PKR is also inhibited at high concentrations of dsRNA (>10 μ g/ml as assessed by in vitro kinase assays. The data also reveal a reduction in IL-4 mRNA levels at high dsRNA concentrations.

Example 14: Cytokine protein expression is induced by dsRNA treatment

Ramos cells, CEM human T cells, and human PBLs were treated with 1 μ g/ml of poly I:C. After 24 hours, the expression of IL-4 and IFN- γ was examined by ELISA (Fig. 14). The error bars indicate the average of two different experiments. These data indicate that dsRNA

- 23 -

induces the production of a Th2-type profile of cytokines at concentrations of approximately 0.01-5 $\mu\text{g/ml}$.

All references cited herein are incorporated by reference in their entirety. While the invention has been described in detail, and with reference to specific embodiments thereof, it will be apparent to one with ordinary skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

10 Other embodiments are within the claims.

- 24 -

What is claimed is:

1. A method of inhibiting an undesired Th1-associated immune response in a mammal suffering from or at risk of developing said response comprising
5 administering to said mammal a substantially pure double-stranded ribonucleic acid (dsRNA).
2. The method of claim 1, wherein said undesired Th1-associated immune response is an autoimmune disease.
3. The method of claim 1, wherein said undesired
10 Th1-associated immune response is tissue transplant rejection.
4. The method of claim 1, wherein said undesired Th1-associated immune response is skin contact dermatitis, Hashimoto's thyroiditis, or Lyme Disease-
15 associated arthritis.
5. The method of claim 2, wherein said autoimmune disease is selected from the group consisting of Grave's disease, multiple sclerosis, Crohn's disease, rheumatoid arthritis, type 1 diabetes mellitus, and juvenile chronic
20 arthritis.
6. The method of claim 5, wherein said autoimmune disease is multiple sclerosis.
7. The method of claim 5, wherein said autoimmune disease is rheumatoid arthritis.
8. The method of claim 1, wherein said dsRNA is
25 at least 10 nucleotides in length.
9. The method of claim 8, wherein said dsRNA is at least 30 nucleotides in length.

- 25 -

10. The method of claim 8, wherein said dsRNA is at least 50 nucleotides in length.

11. The method of claim 8, wherein said dsRNA is at least 100 nucleotides in length.

5 12. The method of claim 1, wherein said dsRNA is derived from a virus or virally-infected cell.

13. The method of claim 10, wherein said virus is selected from the group consisting of a Respiratory Enteric Orphan Virus (reovirus), rhinovirus, vaccinia
10 virus, adenovirus, influenza virus, Polio virus, Epstein-Barr virus, and bacteriophage ϕ .

14. The method of claim 1, wherein said dsRNA is chemically synthesized.

15 15. The method of claim 11, wherein said dsRNA comprises polyribonucleosinic-polyribocytidilic acid (poly I:C).

16. The method of claim 11, wherein said dsRNA comprises polyribonucleosinic acid/polycytidilic acid/uridylic acid (poly(I):poly(C₁₂U)).

20 17. The method of claim 14, wherein said dsRNA is synthetic viral dsRNA.

18. The method of claim 1, wherein said dsRNA is administered systemically.

25 19. The method of claim 18, wherein said dsRNA is administered intravenously.

- 26 -

20. The method of claim 1, wherein said dsRNA is administered locally to a site in the body of said mammal..

21. The method of claim 20, wherein said site is
s an arthritic joint.

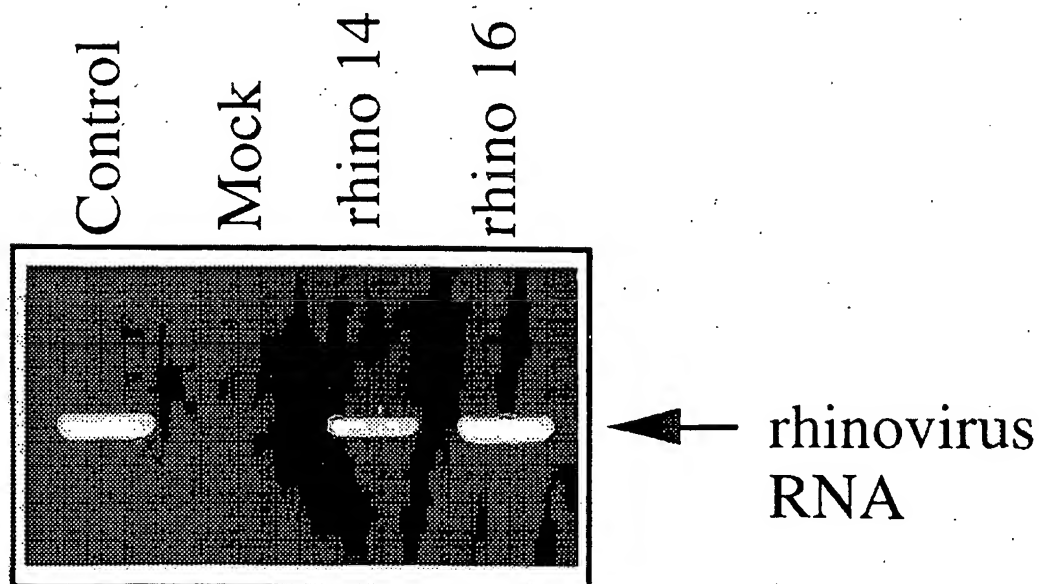
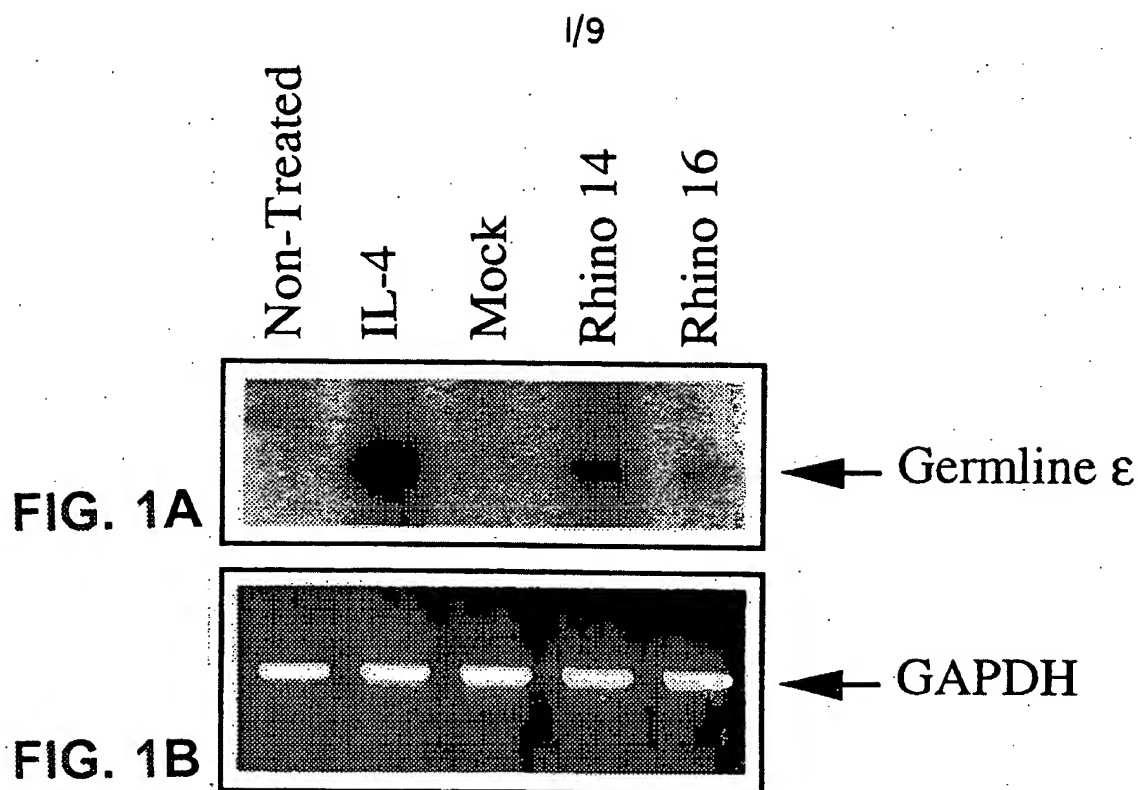


FIG. 2

2/9

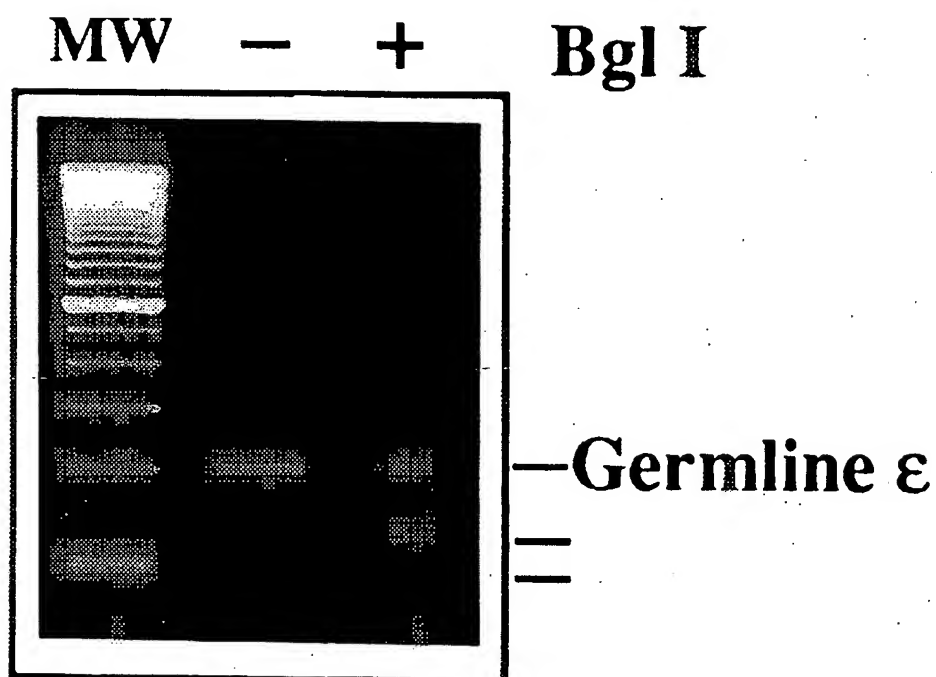
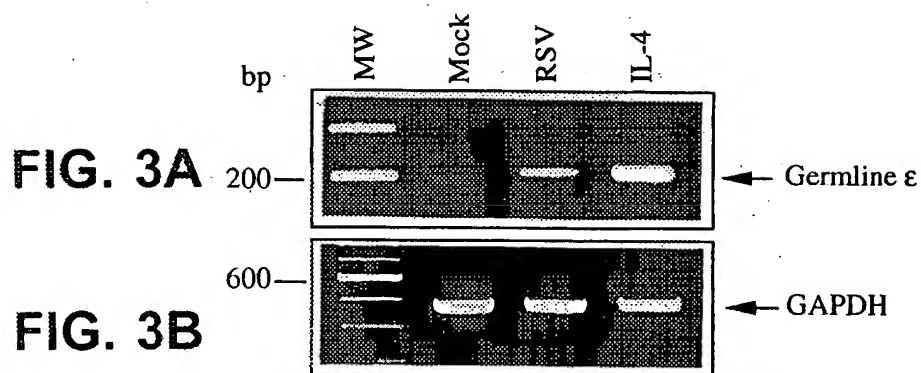


FIG. 4

3/9

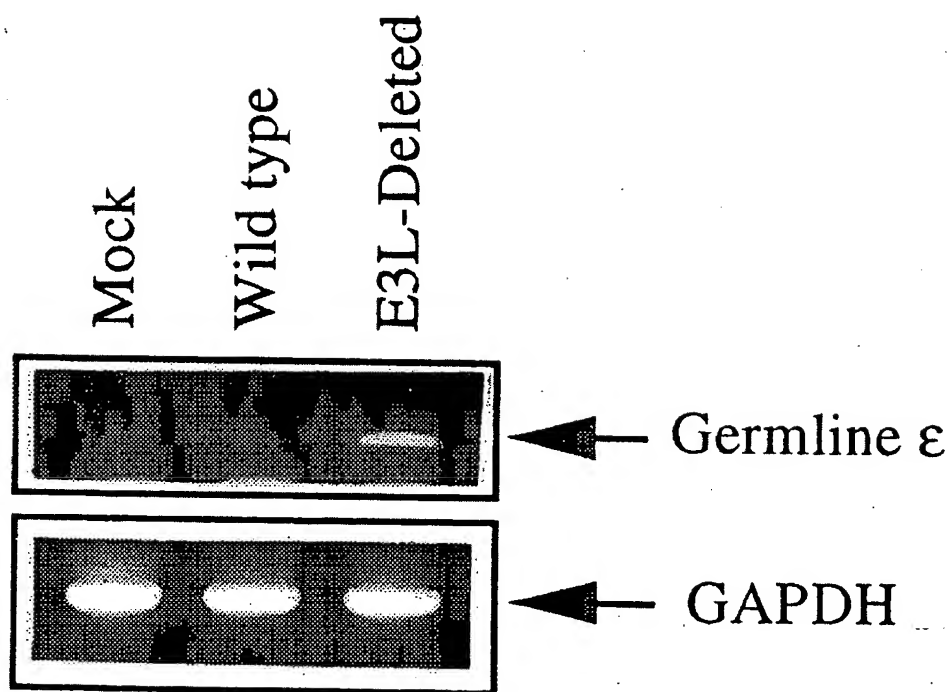


FIG. 5

4/9

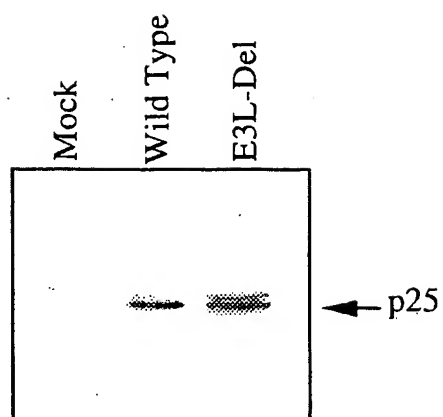


FIG. 6

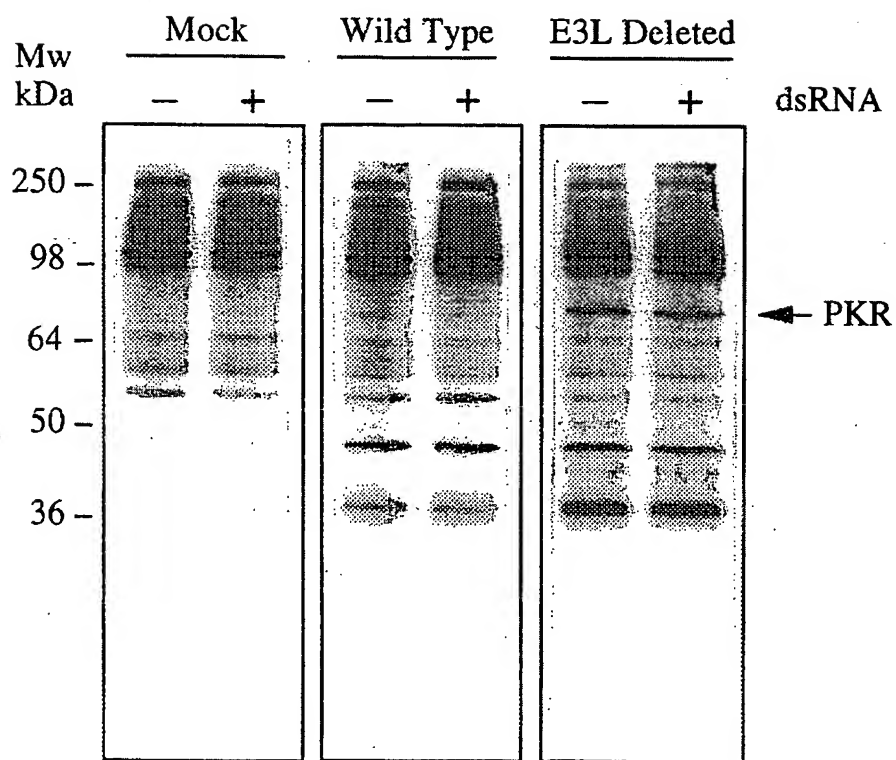


FIG. 7

5/9

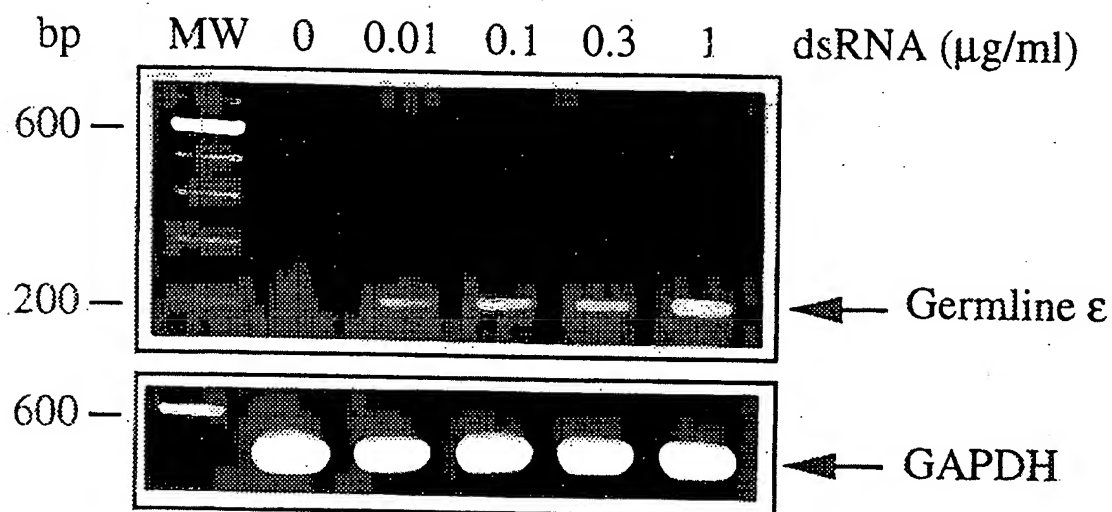


FIG. 8

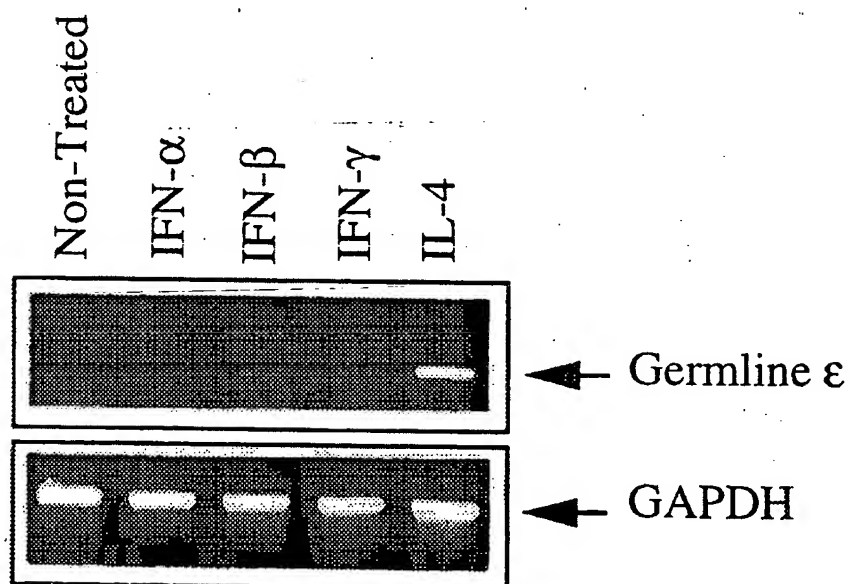


FIG. 9

6/9

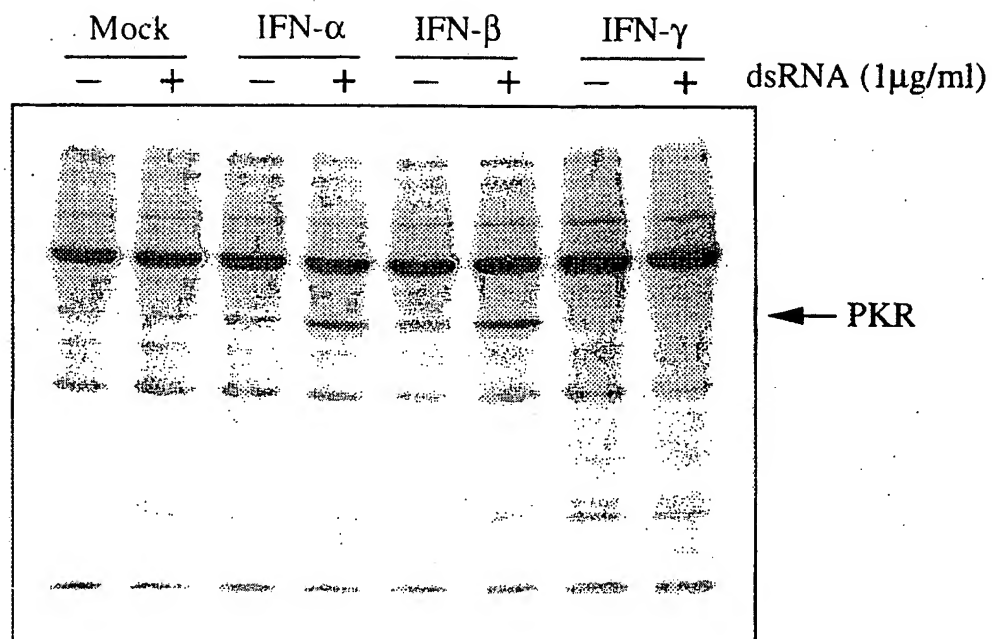


FIG. 10

7/9

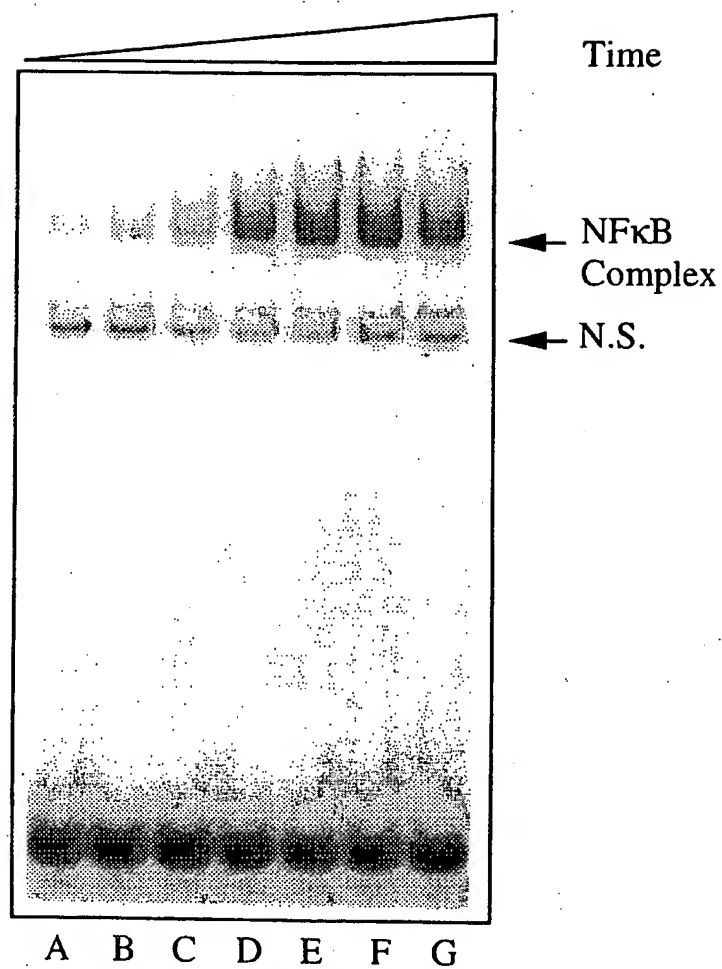


FIG. 11

8/9

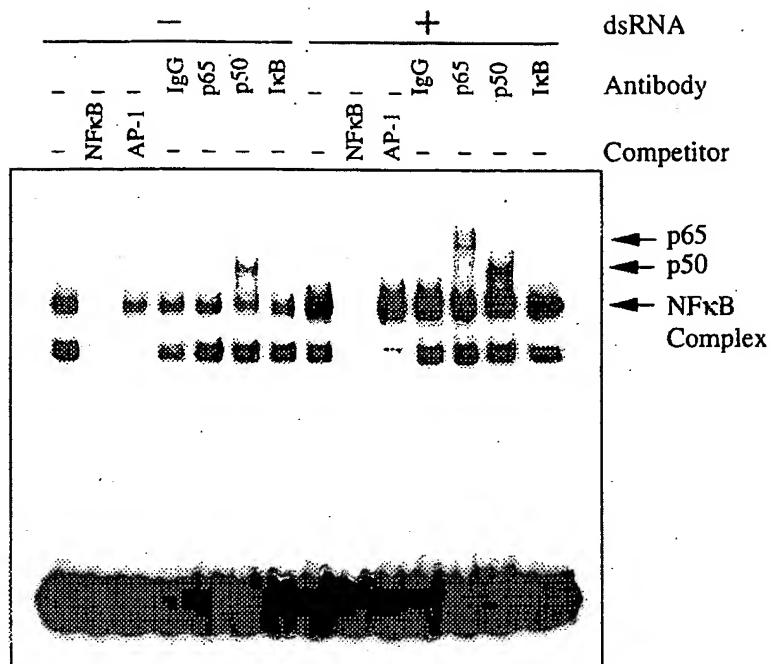


FIG. 12

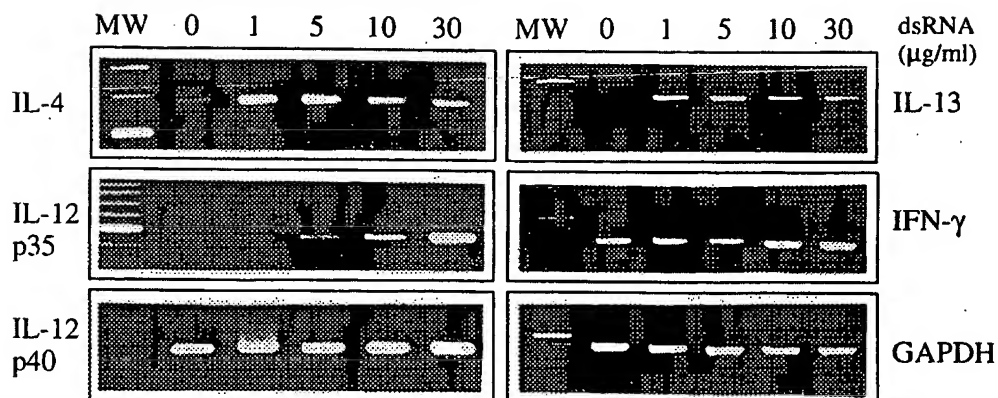
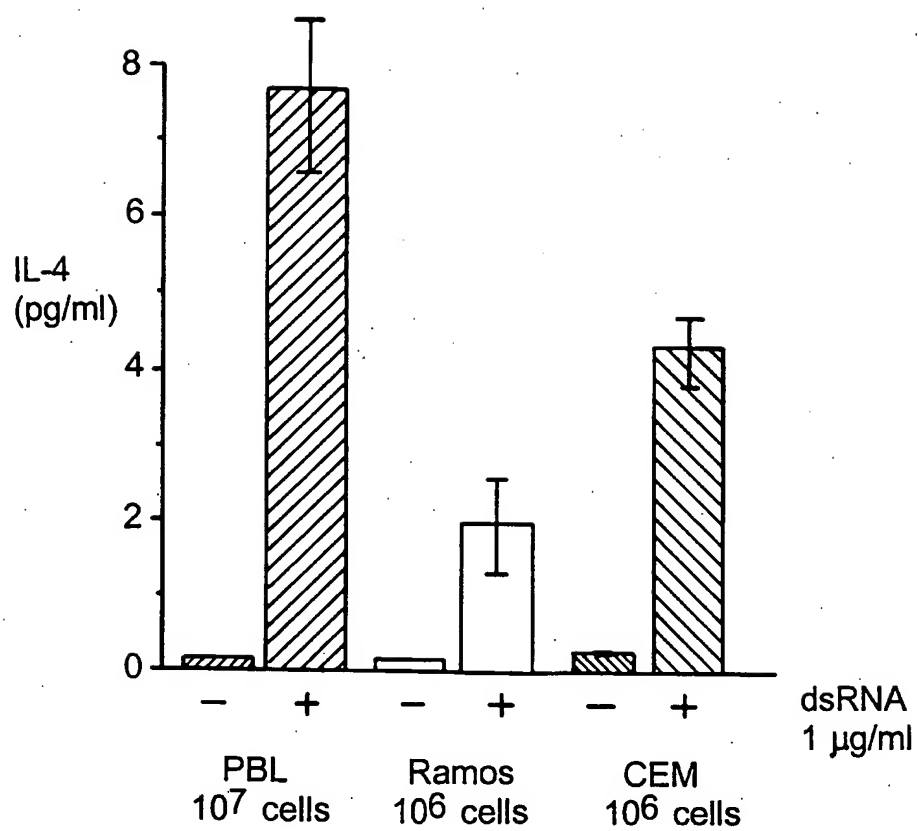


FIG. 13

9/9

**FIG. 14**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/02116

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 48/00; C12Q 1/68; C07H 21/02, 21/04

US CL : 514/44; 435/6, 91.1, 325, 375; 536/23.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/6, 91.1, 325, 375; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
aps, dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,593,973 A (CARTER) 14 January 1997, see entire document.	1, 8, 9, 14
X	US 5,683,986 A (CARTER) 04 November 1997, see entire document.	1, 18
X	US 5,712,257 A (CARTER) 27 January 1998, see entire document.	1, 20
X	US 4,963,532 A (CARTER) 16 October 1990, see entire document.	1

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*a* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

26 MARCH 1999

Date of mailing of the international search report

12 APR 1999

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MARY SCHMIDT

Telephone No. (703) 308-0196

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.